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A Novel Thiazolidinone Herbicide is a Potent Inhibitor of Glucose Incorporation into Cell Wall Material†

Kate R. Sharples, Tim R. Hawkes,* Glynn Mitchell, Lucretia S. Edwards, Mike P. Langford, David W. Langton, Katherine M. Rogers, Jane K. Townson & Youlin Wang

Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET, UK

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Abstract: 5-*tert*-Butyl-carbamoyloxy-3-(3-trifluoromethyl) phenyl-4-thiazolidinone (compound **1**), a representative of a novel class of thiazolidinone herbicides, shows potential for the pre-emergence control of grasses and small-seeded broad-leaved weeds in crops such as soyabean. This compound powerfully inhibited the growth and lateral branching of the roots of agar-grown seedlings of susceptible species but had no apparent effect on the growth of *Escherichia coli*, *Aspergillus nidulans*, *Fusarium culmorum* or of insects cells in liquid culture. It inhibited the growth of plant (*Daucus carota* and *Zea mays*) cells in liquid culture and this inhibition was not reversed by addition to the medium of mixtures of either amino acids or of nucleosides. It did not inhibit fatty acid biosynthesis, respiration, the biosynthesis of sterols, or the biosynthesis of protein in the systems examined herein. By contrast, it induced potent (IC_{50} *c.*50 nM) and rapid inhibition of the incorporation of [^{3}H]glucose into the acid-insoluble cell-wall fraction of roots. Thus, compound **1** exerts its herbicidal effect, directly or indirectly, through inhibition of the biosynthesis of cellulose and cellulose-like polysaccharides, in a manner similar to isoxaben and dichlobenil. Mutant lines of *Arabidopsis thaliana* selected for resistance to isoxaben were cross-resistant to compound **1**. Consistent with the selective herbicidal activity observed, the compound was a potent inhibitor of [^{3}H]glucose incorporation into the polysaccharide of seedling roots of *Zea mays* and of *Setaria viridis* but only relatively weakly active on *Glycine max* and *Ipomoea hederacea*. Given that compound **1** appeared to be metabolised only slowly and that [^{3}H]glucose incorporation experiments were conducted within a total period of less than 90 min, it seems probable that, as in the case of the acetyl CoA carboxylase-inhibitor graminicides, the observed selectivity is determined by species-dependent differences at the (in this case unknown) molecular target site for the herbicide. © 1998 Society of Chemical Industry

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Key words: mode of action; inhibition of glucose incorporation; cellulose biosynthesis; herbicide; 5-*tert*-butyl-carbamoyloxy-3-(3-trifluoromethyl)phenyl-4-thiazolidinone

* To whom correspondence should be addressed.
E-mail: tim.hawkes@aguk.zeneca.com

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1 INTRODUCTION

5-*tert*-Butyl-carbamoyloxy-3-(3-trifluoromethyl)phenyl-4-thiazolidinone (Fig. 1; **1**) is a representative of a novel class of *N*-phenyl-lactam-carbamate herbicides recently reported by us.[1] This compound is an experimental herbicide which shows potential for the selective pre-emergence control of a range of weed species in soybean. Susceptible weeds include grasses (such as *Digitaria spp.*, *Setaria spp.*, *Sorghum spp.*, *Bachiaria spp.* and *Echinochloa crus-galli* Beauv.) and small-seeded broad-leaved weeds (including *Amaranthus spp.* and *Chenopodium spp.*), though large-seeded broad-leaved weeds (such as *Ipomoea spp.* and *Abutilon spp.*) are not controlled. The compound shows excellent crop selectivity at the rates required for control of susceptible weed species.

In this paper, we describe the results of a study designed to elucidate the mode of action of **1**, and the basis of its differential activity towards susceptible and non-susceptible plant species.

2 MATERIALS & METHODS

2.1 Chemical synthesis and preparation of solutions for testing

The method used for the synthesis of **1** is outlined in Fig. 1. Stage 1 involves the cyclocondensation of 3-trifluoromethylaniline, thioglycolic acid and formaldehyde to form the thiazolidinone ring. Stage 2 comprises an initial chlorination at the thiazolidinone 4-position, followed by hydrolysis to the corresponding alcohol. Stage 3 involves reaction of this alcohol with *tert*-butyl isocyanate to install the carbamate side chain. Full preparative details of each stage of the process have been reported previously.[1]

Concentrated stock solutions in dimethyl sulfoxide (DMSO) were prepared and diluted into the test media described in Sections 2.2–2.8 such that the final concentration of DMSO did not exceed 2 ml litre^{-1}. Stock solutions of other chemicals that were used as standard inhibitors in individual tests were prepared similarly. Control experiments contained DMSO alone.

2.2 Tests for herbicidal activity

The pre-emergence herbicidal activity of **1** was determined under glasshouse conditions (24/19°C day/night with a 14-h photoperiod, supplementary lighting provided by metal halide lamps). The seeds were sown in a loamy sand (1·6% organic matter content, pH 7) at a depth of 1 cm for weeds and 2 cm for crop species. Plant species included *Setaria viridis* Beauv., *Echinochloa crus-gallii* Beauv., *Sorghum halepense* Pers., *Chenopodium album* L., *Amaranthus retroflexus* L., *Ipomoea hederacea* Jacq., *Abutilon theophrasti* (L.) Medic., *Glycine max* Merr. and *Zea mays* L. The seeds were watered two hours before spraying and lightly watered afterwards. The compound was formulated in 4% JF4400 (21·8 g litre^{-1} Span 80, 78·2 g litre^{-1} Tween 20 made up to 1 litre with methyl cyclohexanone) and the plants were sprayed (8001E nozzle, 400 litre ha^{-1} at 2 psi) at a range of application rates. The plants were assessed 20 days after treatment and ED_{50} values were estimated for each species.

Compound **1** was also tested for activity in field trials at Zeneca's field stations at Rocky Mount, Leland and Champaign in the USA, under well-irrigated conditions. Test species included *Digitaria sanguinalis* Scop., *E. crus-gallii*, *Setaria faberi* Herrm., *S. halepense*, *Sorghum vulgaris* Pers., *A. retroflexus* and *I. hederacea*.

2.3 Assay for inhibition of growth of seedlings on agar

Test compounds at various concentrations (typically 0·03–30 μM) were incorporated into Murashige–Skoog

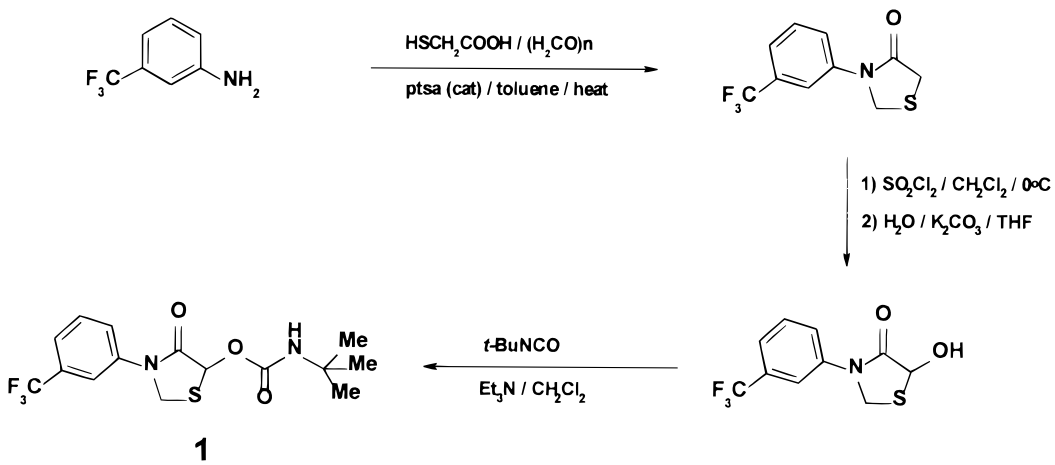

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Fig. 1. Synthesis of 5-*tert*-butyl-carbamoyloxy-3-(3-trifluoromethyl)phenyl-4-thiazolidinone (compound **1**).

basal medium solidified with agar (Phytagel). Sterilised wild-type seeds of *S. viridis*, *I. hederacea*, *G. max* and *Z. mays* were sown onto the agar surface and root and shoot lengths were measured after six days.

Seeds of the isoxaben-resistant *Arabidopsis thaliana* Heynh. line Ixrl-1 were obtained from the Arabidopsis Biological Resource Centre, Ohio State University, USA.[2] This line and wild-type *A. thaliana* were treated as above and assessed visually for inhibition of growth after 15 days.

2.4 Growth of plant cells in liquid culture: inhibition and reversal

Black Mexican Sweetcorn (*Z. mays*) cells were grown in Murashige–Skoog medium containing 2,4-dichlorophenoxyacetic acid (2,4-D; 2 mg litre^{-1}). *Daucus carota sativa* Arang. line DC3 was maintained in the same medium but containing 2,4-D and kinetin (0·1 and 0·2 mg litre^{-1}, respectively). Stock lines were subcultured every seven days. Growth experiments were started from a 10% inoculum into medium (10 ml) kept gently rotary-shaken in 25-ml conical flasks. Controls (zero growth) contained a powerful standard inhibitor (e.g., a sulfonyl urea herbicide). After five days the cells were freeze-dried and weighed. Attempts to reverse inhibition of growth were made by including casein hydrolysate (2·5 mg ml^{-1}), 2-ketoglutarate (0·6 mg ml^{-1}) or mixtures of adenosine, guanosine, cytidine and uridine (0·3 mg ml^{-1} each) in the medium. In control experiments these additions appropriately reversed the inhibitory effects of sulfometuron-methyl (an inhibitor of acetolactate synthase),[3] acetylmethylphosphinate (an inhibitor of pyruvate dehydrogenase)[4] and hadacidin (an inhibitor of adenylosuccinate synthase),[5] respectively.

2.5 Growth of bacteria, fungi and insect cells

Escherichia coli Castell. & Chalm. strain JM101 was seeded as a lawn onto a plate of agar made up in minimal medium containing glucose as sole carbon source, and 50 µl of a solution of test-compound was added to small wells cut into the agar. Zones of bacterial growth-inhibition around these wells were assessed following overnight growth at 37°C. Agar plugs of the fungi *Aspergillus nidulans* (Eidam) Winter and *Fusarium culmorum* Sacc. were applied to plates of Luria–Bertani medium solidified with agar made up with and without test compound. The diameter of mycelial growth was measured after 60 h and six days at 20°C.

Sf21 insect cells were grown in TC100 (GIBCO) medium supplemented with 5% heat-inactivated foetal calf serum in microtitre wells and grown for four days at 27°C, either in the presence or absence of 70 µM of test compound. Then 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyltetrazolium bromide (MTT; 1 mg ml^{-1}) was added to each well and incubated for 4 h. After decanting the supernatant, the residue of cell layer at the bottom was then dispersed evenly in DMSO and the colour read at 540 nm using a Dynatech plate reader.

2.6 [^{14}C]Acetate incorporation into sterol and fatty acid components of *Zea mays* cells

Test compound was added to maize cells growing in liquid culture as described in Section 2.4. After 30 min, 55·5 kBq (0·5 mM) of sodium [^{14}C]acetate was added and the mixture stood for 10 min, after which the cells were rapidly chilled by transfer to ice, isolated from the medium by vacuum filtration onto a filter paper and washed with 10 mM sodium acetate before disruption and extraction into acidified isopropanol + chloroform (1 + 2 by volume). After low-speed centrifigation, some of the chloroform layer was removed and evaporated under nitrogen. The lipid residue was re-dissolved in chloroform (1 ml) and 50 µl of this solution was loaded onto a 0·25-mm silica TLC plate, and chromatographed using hexane + diethyl ether + acetic acid (80 + 20 + 1, by volume) as eluent. After drying, the TLC plate was scanned using a FujiBAS 1500 phosphor imaging system to produce a chromatogram of the incorporated radiolabel. The main peaks corresponded to phospholipids (R_f *c.*0·04), glycolipids (R_f *c.*0·08), sterols (R_f *c.*0·13 and 0·16), fatty acids (R_f *c.*0·44) and sterol esters (R_f *c.*0·83).

2.7 Assay for changes in the profile of plant sterols

Lipid was extracted from *Z. mays* cells treated for three days with 0 (control), 10 or 50 µM test compound, exactly as described in Section 2.4. Chloroform aliquots (50 µl) were derivatised with *bis*(trimethylsilyl)trifluoroacetamide and loaded onto a Hewlett Packard 5890 series gas chromatograph fitted with a Hewlett Packard ultra 1 (12 m × 0·2 µm) column. The resulting chromatogram was analysed for sterols using a Hewlett Packard 5971 mass selective detector. Sterols were identified by comparing the mass spectra with those of standard plant sterols.[6]

2.8 Assay for inhibition of DNA-directed protein synthesis in *Escherichia coli* extracts

The method used was that described by Zubay,[7] slightly modified and used as described by Hawkes *et al.*[8] for detection of inhibitors. The DNA used was pGEM-βgal

(lac Z) from Promega. Test reactions contained 30 µl of 'S30' *E. coli* extract, 15 µl of concentrated reagent solution and 5 µl of test compound. Herbicidal inhibitors of plastidic protein biosynthesis, such as esters of monic acid, which inhibit isoleucyl tRNA synthetase[9] were readily detected as potent inhibitors at <5 µM.

2.9 Assay for incorporation of [^{3}H]glucose into acid-insoluble polysaccharide

Seeds were germinated for three to five days between layers of tissue paper and sand. Samples (20 mm long) were cut from the root tips of *Z. mays*. *I. hederacea* and *G. max* seedlings and bulked into 0·3-g lots which were placed in 15-ml glass vials containing White's root culture medium (10 ml) at 25°C. Whole seedlings of *S. viridis* were used and bulked up into 0·4-g lots in vials as above. Test compounds were added at a range of concentrations (0·001–25 µM) and incubated with gentle rotary shaking for 20 min, then [^{3}H]glucose (74 kBq) was added and each tube gently rotary-shaken for a period of 1 h. Incorporation was stopped by raising the temperature to 100°C for 5 min. 'Zero' incorporation controls were killed by pre-heating to 100°C for 5 min. Plant material was washed twice *in situ* with water and then homogenised for 30 s in DMSO + water (90 + 10 by volume) using an Ultraturrax, and de-starched through further incubation in DMSO for 2 h at 95°C. Samples were then collected by low-speed centrifugation, washed in water, spun again, taken up in trifluoroacetic acid (2 M; 5 ml) and incubated at 110°C for 2 h. Samples were cooled, collected on paper filter papers, washed, dried, combusted (Packard Oximate 80) and counted using a liquid scintillation counter (Beckman LS 6000TA). To validate the test system it was confirmed that incorporation was near linear with time over at least 2 h.

3 RESULTS AND DISCUSSION

3.1 Herbicidal activity of compound 1

Representative ED_{50} values from the glasshouse tests are shown in Table 1. These results clearly demonstrate that the grasses (*S. viridis*, *E. crus-gallii*, *S. halepense*) and small-seeded broad-leaved species (*C. album*, *A. retroflexus*) are much more sensitive to **1** than are the large-seeded broad-leaves species (*I. hederacea*, *A. theophrasti*). The results also show that *G. max* shows a significantly greater degree of tolerance towards **1** than does *Z. mays*.

In the field, **1** gave complete control of several weed species, as shown in Table 2. No damage to soybean was observed at application rates of 1000 g ha^{-1}. Some suppression of *I. hederacea* was seen in some trials at high application rates (500–1000 g ha^{-1}), though complete control was not achieved.

TABLE 1
Herbicidal Activity of Compound **1** in the Glasshouse

Species	ED_{50} (*g ha*$^{-1}$)
Glycine max	*c.*20 000
Zea mays	770
Setaria viridis	54
Echinochloa crus-gallii	87
Sorghum halepense	113
Chenopodium album	16
Amaranthus retroflexus	19
Ipomoea hederacea	*c.*3500
Abutilon theophrasti	830

3.2 Clues to the mode of action of compound 1 from biological symptoms

Compound **1** caused severe stunting of growth and complete inhibition of seed germination when used at high rates. Some of the more tolerant species, such as *I. hederacea*, exhibited 'sub-lethal' effects which included swelling and splitting of stems, but the most profound effects were observed on roots. In agar, the roots of treated *S. viridis* developed as brown, stunted, swollen and without lateral branches or root. Root growth was completely inhibited at a concentration of *c.*2 µM in agar. Visually, the symptoms induced in roots appeared somewhat distinct from those induced by dinitroaniline herbicides which act through binding to tubulin.[10]

Some further possible modes of action were immediately recognised as unlikely on the basis of the biological symptoms observed. Compound **1** did not bleach plants (and thus was unlikely to inhibit the biosynthesis of carotenoids), nor did it cause rapid scorching (and thus was unlikely to act in a similar way to inhibitors of protoporphyrinogen oxidase or redox mediators of photosystem I). The pre-emergence activity and weed spectrum were reminiscent neither of inhibitors of photosystem II, nor of hormone (2,4-D) type herbicides.

TABLE 2
Application Rates Required for Weed Control in the Field

Species	*Application rate* (*g ha*$^{-1}$)
Echinochloa crus-gallii	250–500
Sorghum halepense	250
Sorghum vulgaris	250
Digitaria sanguinalis	125–250
Setaria faberi	125–250
Amaranthus retroflexus	63–125

3.3 Effect of compound 1 on growth of bacteria, fungi, insect and plant cells in liquid culture

At a nominal concentration of 0·8 mM, **1** did not detectably inhibit the growth of *E. coli*. Similarly at 0·3 mM, it only slightly ($< 20\%$) reduced the growth rates of *A. nidulans* and of *F. culmorum* mycelium, but had no effect on either appearance or final biomass after six days. At 0·07 mM, **1** had no effect on either the growth or viability (as judged by the reduction of MTT dye) of Sf21 insect cells. This test is a sensitive indicator for general respiratory uncouplers of oxidative phosphorylation and also for other metabolic toxic effects such as inhibition of respiration and inhibition of dihydrofolate reductase.[11] At 1, 10 and 100 μM, compound **1** inhibited the growth of *D. carota* cells by 24, 46 and 52% relative to controls; *Z. mays* cells were similarly inhibited by 52% at 100 μM.

Herbicidal molecules which act by inhibiting the major pathways of, for example, the biosynthesis of amino acids or of purines can often be recognised on the basis of the fact that the inhibitory effect which they exert on the growth of plant cells can be specifically reversed by addition of the end-product of the inhibited pathway. However, over a range of treatments, the inhibitory effect of **1** on growth was undiminished in the presence of casein hydrolysate, 2-ketoglutarate or various mixtures of nucleosides. Whilst falling short of absolute proof, these experiments strongly suggest that it is unlikely that the herbicidal activity of **1** is due to inhibition of the biosynthesis of amino acids, purines or pyrimidines.

3.4 [^{14}C]Acetate incorporation into fatty acids and sterols

In untreated *Z. mays* cells the relative percentages of incorporation of [^{14}C]acetate were as follows: phospholipids (69); glycolipids (5); sterols (8·5); fatty acids (12) and sterol esters (6). Treatment of cells for 30 min with either 50 μM fluazifop acid (a standard herbicide which inhibits acetyl CoA carboxylase)[12] or methyl (*E*,*E*)-α-(methoxymethylene)-2-(2-phenylethenyl)phenylacetate (a strobilurin-derived inhibitor of respiration)[13] resulted, in both cases, in a *c*.80% decrease in total incorporation and a shift to a disposition of percentage incorporation to: phospholipids (36); glycolipids (4); sterols (40); fatty acids (0·2); sterol esters (20). Under similar conditions, mevinolin (a potent inhibitor of HMGCoA reductase)[14] resulted in a 25% decrease in total incorporation and a shift to a percentage disposition of phospholipids (83); glycolipids (5); sterols (0); fatty acids (5) and sterol esters (0). By contrast, treatment with **1** at a nominal concentration of 200 μM for more than 2 h had no discernible effect on either the total level of incorporation or the disposition of incorporation of [^{14}C]acetate incorporation into the lipid fractions. Furthermore, consistent with its lack of cytotoxicity versus Sf21 cells, **1** did not uncouple oxidative phosphorylation or inhibit respiration in intact mitochondria extracted from cockroach flight muscle. Thus, **1** did not inhibit fatty acid biosynthesis, sterol biosynthesis or respiration. These conclusions were further substantiated by data from in-vitro studies (Hawkes, T. R., unpublished), indicating that **1** did not inhibit maize acetyl CoA carboxylase or the incorporation of [^{14}C]malonyl CoA into palmitate by spinach stromal fatty acid synthase.

3.5 Sterol profile of *Zea mays* cells

The relative percentages of campesterol, stigmasterol and sitosterol in control maize cells were 13, 33 and 54, respectively. Treatment with 10 μM of a γ-keto triazole herbicide known to inhibit obtusifoliol ^{14}C-demethylase[15] resulted in massive changes, with the stigmasterol and sitosterol peaks being reduced to *c*.5% and 22%, respectively, and the campesterol peak being more or less abolished and replaced by a new peak, identified as 14-α-methyl-24-dihydrofecosterol (with characteristic mass peaks at *m*/*e* 486, 471, 381, 227, 213 and 196) and accounting for *c*.70% of the total sterol. Treatment for three days with 10 μM **1** resulted in no discernible effect on the sterol profile of either maize or carrot cells. Thus **1** did not, apparently, exert its herbicidal effect through interference with the later steps of plant sterol biosynthesis.

3.6 DNA-directed protein synthesis in extracts of *Escherichia coli*

The machinery of protein biosynthesis within the plastid is broadly similar to that within eubacteria. DNA-directed protein synthesis in *E. coli* extracts can serve as a proxy for protein and RNA biosynthesis within the plastid,[16] and thus, for example, inhibitors of isoleucyl tRNA synthetase which are both antibiotics and herbicides can be readily detected through inhibition of this process.[9] However, in this case, even at 20 μM; **1** had no detectable effect on the rate of in-vitro synthesis of β-galactosidase directed by plasmid DNA.

3.7 [^{3}H]glucose incorporation into acid-insoluble polysaccharide

A series of experiments was conducted to examine the effect of **1** on the incorporation of [^{3}H]glucose into the acid-insoluble polysaccharide cell wall fraction of *Z. mays* roots, usually assumed to be cellulose.[17] The results are summarised in Fig. 2. Compound **1** was a potent inhibitor of glucose incorporation, similar to isoxaben (*N*-(3-[1-ethyl-1-methylpropyl]-5-isoxazoloyl)-2,

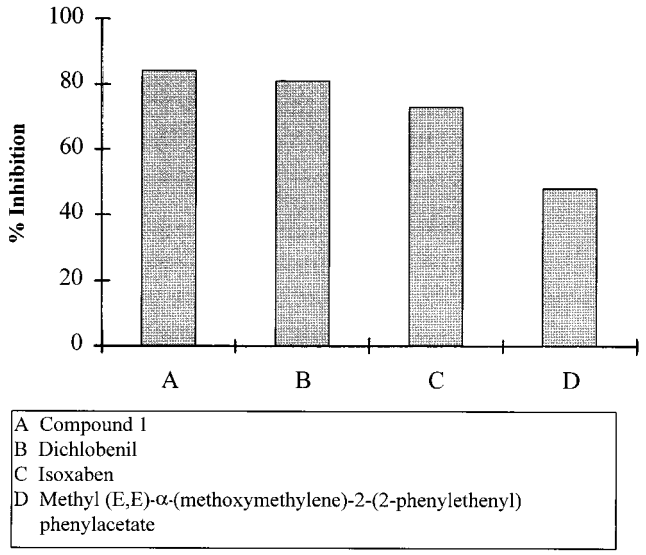

Fig. 2. Inhibition of cellulose biosynthesis by test compounds (10 μM) in seedling roots of *Zea mays.*

6-dimethoxybenzamide)[18] and dichlobenil (2,6-dichlorobenzonitrile).[19] Experiments using a range of standard metabolic inhibitors and herbicides confirmed that inhibition was not a general phenomenon but was peculiar to **1**, isoxaben and dichlobenil (Wang Y., unpublished; see also Reference 18). The exception was the respiration inhibitor, methyl (*E,E*)-α-(methoxymethylene)-2-(2-phenylethenyl)phenylacetate, which also exerted a significant—and presumably less direct—inhibitory effect. As discussed above, **1** is not an uncoupler of oxidative phosphorylation, and does not inhibit respiration. Thus, like isoxaben and dichlobenil, **1** acts directly or indirectly to inhibit cellulose biosynthesis. The IC_{50} value for inhibition was *c.*50 nM, with a maximum level tending to approximately 90% at high treatments. It is, of course, difficult to judge how such apparent IC_{50} values might translate to real binding constants at the (unknown) molecular target because of the confounding effects of uptake and partition. However the value clearly reports on a system which has come to equilibrium since (i) it was not highly dependent on time, as similar values were obtained from experiments conducted over 1 or 2 h, and (ii) some analogues of **1** yielded much lower values (Wang, Y., unpublished), indicating that the value is useful and not merely an arbitrary lower limit set by the concentration of target site within a particular root preparation.

Further biological evidence for **1** having an action on cell wall biosynthesis came from a closer examination of the inhibition of growth of plant cell culture under the light microscope. Sub-lethal treatments of **1**, isoxaben and of dichlobenil all caused a characteristic swelling of tobacco BY2 cells (up to three-fold after five days) which was not apparent with herbicides such as trifluralin and acetochlor which have different modes of action. Inhibition thus appeared much less when assessed on the basis of the settled volume of wet cells than on the basis of dry weight.

3.8 An isoxaben-resistant *Arabidopsis* mutant is cross-resistant of compound 1

The results from this study into the mode of action of **1** were very similar to those reported by Heim *et al.*[18] for the herbicide isoxaben. Using *A. thaliana* plants, these workers observed that isoxaben had no effect on the incorporation of radio-labelled precursors into protein, nucleic acids or fatty acids, but potently and specifically (and unlike a range of control metabolic inhibitors) inhibited the incorporation of radio-labelled glucose into the acid-insoluble fraction of the cell wall. As was found with **1**, isoxaben was more active against root tissue than against cells in culture and, again, activity was in the nM range with a maximum degree of inhibition that was less than complete (close to 80%).

The mutant Ixr1-1, which is resistant to isoxaben,[2] is cross-resistant to **1** (Fig. 3). This provides some direct evidence for **1** and isoxaben sharing the same molecular target (which is not shared with dichlobenil).[20] Isoxaben at 0·3 μM killed wild-type *Arabidopsis* whilst only reducing the growth of the resistant biotype by 20% (Fig. 3). Thus the resistant biotype had at least a five-fold tolerance to isoxaben. At 12 μM, compound **1** killed the wild-type *Arabidopsis* but reduced the growth of the resistant biotype by only 50%. Thus the resistant

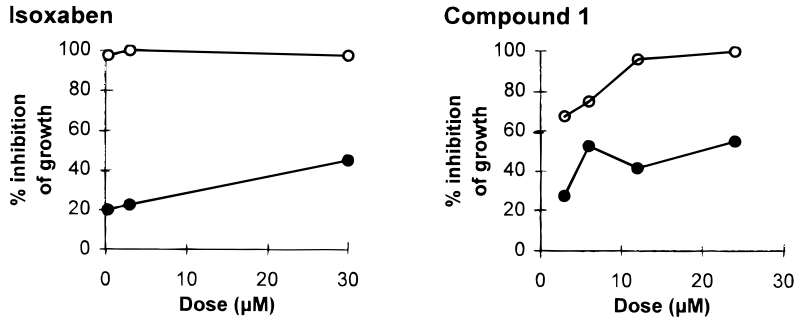

Fig. 3. The effect of isoxaben and compound **1** on the growth of (○) wild-type and (●) resistant *Arabidopsis thaliana.*

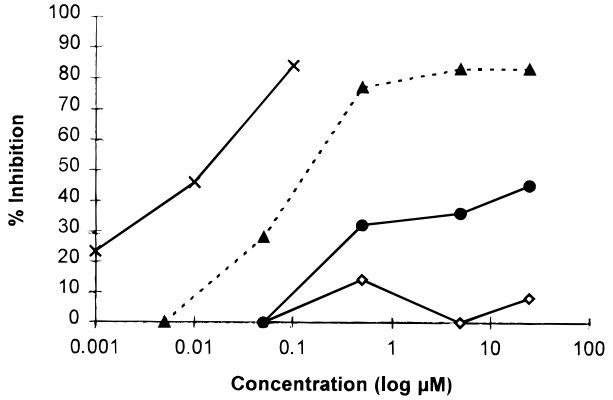

Fig. 4. The inhibition of cellulose biosynthesis by compound **1** in roots of (◇) *Glycine max*, (▲) *Zea mays* and (●) *Ipomoea hederacea*, and (×) in whole seedlings of *Setaria viridis*.

biotype was also significantly (at least two-fold) resistant to **1**.

3.9 Species-dependent differences in inhibition of [^{3}H]glucose incorporation reflect differences in the herbicidal activity of compound 1

The inhibition of glucose incorporation into the acid-insoluble cell-wall material is clearly species-dependent, as demonstrated in Fig. 4. In *S. viridis* and *Z. mays*, glucose incorporation was inhibited by 80% at concentrations $>0{\cdot}1$ and >1 μM, respectively, whereas significantly higher treatments were required to produce the same level of inhibition in *I. hederacea* and *G. max*. These results closely parallel the observed herbicidal activity of **1** against three species (see Table 1 for ED_{50} values).

The rate of metabolism of **1** in plants was readily measured as loss of parent compound after addition to *G. max* cell culture. The compound was identified by HPLC using a Hi-chrom, C18, ODS2 reverse-phase column. A mobile phase of acetonitrile + water (65 + 35 by volume) was used and detection was by UV at a wavelength of 210 nm. There was no significant loss of parent compound after 24 h. Further tests using radiolabelled close analogues of **1** demonstrated that, in general, this class of herbicide is not metabolised in *G. max* for at least three days after application (Hayhurst I., unpublished). This slow metabolism, combined with the relatively short period over which incorporation assays were conducted, suggests that it is most likely that the differential activity of **1** is directly determined by species-dependent variability at an (unknown) molecular target. Also, as (embryo-derived) maize cell culture seemed markedly less sensitive to inhibition than did maize roots, it seems likely that variability at the molecular target site might also be tissue- as well as species-dependent, and, perhaps, account for the more severe effects of **1** on roots than on shoots and leaves.

4 CONCLUSIONS

Taken together, the experiments described above and the symptoms produced by **1** on plants suggest very strongly that its herbicidal action is due solely to inhibition of cellulose biosynthesis. However the molecular target itself remains to be defined. Little is known of the cellulose-biosynthesising machinery in higher plants. Electron microscope evidence suggests that synthesis occurs within an enzyme complex associated with the plasma membrane, but the nature of this remains obscure, since, thus far, it has proved impossible to isolate without loss of activity.[21] Recent progress in plant genomics will perhaps expedite the identification of its various components.[22] UDP-Glucose has been implicated as the activated form of glucose which acts directly as substrate for catalytic polymerisation and a membrane-associated isoform of sucrose synthase (rather than UDP glucose pyrophosphorylase) seems the most likely candidate to provide this.[23] In principle, **1**, isoxaben and dichlobenil could bind directly to any essential part of the cellulose synthase complex or, alternatively, could act indirectly through interference with regulation or some essential post-translational processing. Whatever the mechanism, it must take effect rapidly since inhibition is expressed within less than an hour of contact with the herbicide (and probably faster, this estimate being limited by our inability to make the measurement more quickly). Certainly none of the herbicides bears any obvious structural resemblance to UDP-Glucose and neither isoxaben nor dichlobenil inhibits the relatively well-characterised cellulose synthase from *Acetobacter xylinum*.[24] It seems most probable that all of the herbicides bind at a site other than the catalytic site and which is in some way unique to the plant system.

There is no evidence that the herbicides need bind at the same site, and indeed there is evidence to the contrary. There are three known isoxaben-resistant mutants of *A. thaliana*. Two confer a high level of resistance and represent different alleles at the same locus (Ixr A)[2] whilst the third confers only a low level of resistance and maps to a locus genetically unlinked to the other two (Ixr B).[25] In all of these mutants cellulose biosynthesis was identified as the primary site of resistance and resistance could not be attributed to differences in uptake or metabolism. The mutants mapped to two independent genes, so it is assumed that isoxaben either acts directly on the putative cellulose synthase complex, which consists of at least two different subunits, or that the genes may encode proteins involved in regulation or post-translational modifications of active site.[26] These mutants were not cross-resistant to dichlobenil and neither were dichlobenil mutants cross-resistant to isoxaben.[20] A dichlobenil photo-affinity analogue was demonstrated to bind to an 18 kD protein not associated with the plasma membrane.[27]

The isoxaben-resistant *Arabidopsis* used in this study had the IxrA mutation and was cross-tolerant to **1**. Thus, isoxaben and **1** probably share at least part of a binding site. It is interesting to note the parallels with quinclorac, which also appears to inhibit the biosynthesis of plant cell walls and where, again, selectivity between species is inferred to be due to differences at an unknown molecular site of action. Quinclorac inhibits the biosynthesis of both celluloses and hemicelluloses and apparently acts at a site distinct from isoxaben and **1**.[28,29]

Consistent with the observation that both isoxaben and **1** never inhibited cellulose biosynthesis by more than 80–90%, and with the observed tissue- and species-selective effects, there is also molecular evidence for there being more than one cellulose-synthesising machinery in plants. There are *Arabidopsis* mutants which are unable to synthesis cellulose only in specific tissues, indicating that there may be different forms of the enzyme in different parts of the plant and that the cellulose required for primary and secondary cell walls may also be differentially regulated.[30–32] Progress in the final identification of the binding site for **1** will most probably require a combination of molecular approaches (e.g. map-based cloning of the resistant mutants) and traditional biochemistry (e.g. identification of the binding site through synthesis of a suitable photo-affinity label).

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